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(54) Title: A METHOD AND REAGENT COMBINATION FOR THE DIAGNOSIS OF MICROORGANISMS BY SANDWICH HYBRIDIZATION OF NUCLEIC ACIDS		
(57) Abstract Microbial diagnostic method based on a sandwich hybridization of nucleic acids on a solid carrier, as well as a reagent combination for utilization in the method. The method described in the invention can be used to identify, from a single sample containing nucleic acids of the microbes or microbial groups to be diagnosed, after first rendering the said nucleic acids single-stranded, all the microbes or microbial groups by adding to the sample two nucleic acid reagents for each microbe or microbial group to be diagnosed, of which one nucleic acid reagent is attached to a solid carrier in the single-stranded form and the other contains a completely different nucleic acid fragment, from the same microbe or microbial group, which is labelled with an established marker. The recognizing nucleic acid fragment hybridizes to a complementary single-stranded nucleic acid from the sample and the hybrid thus formed on the particulate carrier becomes labelled when the complementary labelled nucleic acid fragment anneals to the single-stranded nucleic acid originating from the sample. Because the labelled nucleic acid reagents do not alone hybridize with the solid carrier, only those carriers containing nucleic acid reagents corresponding to nucleic acids in the sample become labelled. The label is measured using established methods.		

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A METHOD AND REAGENT COMBINATION FOR THE DIAGNOSIS OF MICROORGANISM
BY SANDWICH HYBRIDIZATION OF NUCLEIC ACIDS

5 The invention relates to a method for the diagnosis of microbes based on sandwich hybridization of nucleic acids to a solid carrier and to the combination of reagents used in the method.

10 In traditional microbial diagnostics the presence of a microbe in a given sample is demonstrated by isolation of the microbe in question. After enrichment cultivation the microbe is identified either on the basis of its biochemical properties or using immunological methods. This type of diagnosis requires that the microbe in the sample is viable.
15 Identification by isolation is moreover a laborious method, which in the case of viruses may require 4 - 6 weeks.

20 The purpose of this invention is to provide a diagnostic method in which the presence of a microbe in a sample is demonstrated by the identification of its genetic material, the nucleic acid, with the aid of the sensitive and specific nucleic acid hybridization technique. In itself, nucleic acid hybridization is an old and well known method for investigating the identity of nucleic acids. Complementary
25 nucleic acid strands have the ability to form a tight double-stranded structure according to the rules of base pairing, and the resulting hybrid can be separated from the residual single-stranded nucleic acid.

30 Some methods based on the identification of nucleic acid(s) have already been applied to microbial diagnostics. Enterotoxigenic Escherichia coli have been identified from faecal samples by colony hybridization using the gene for toxin production as a probe. Positive hybridization is
35 demonstrated by autoradiography (Moseley, S.L. et al., J. Infect. Dis. (1980) 142, 892-898). Colony hybridization is

based on the method originally developed by Grünstein and Hogness (Proc. Natl. Acad. Sci. USA (1975) 72, 3961-3965). Hybridization has also been used as a method to distinguish between Herpes simplex virus type 1 and type 2 (Brautigam, A.R. et al., J. Clin. Microbiol (1980) 12, 226-234), however, not in rapid diagnostics but in typing of the virus after enrichment cultivation. In this method the double-stranded hybrid is separated from the fraction of nucleic acids remaining single-stranded in the solution by affinity chromatography.

- It was recently published that DNA from cells infected with Epstein-Barr virus (the sample) was after appropriate pretreatment fixed directly onto the filters. The nucleic acid in question is identified by hybridizing the filters with the radioactive probe and positive hybridization is detected by autoradiography (Brandsma, I. and Miller, K. (1980) Proc. Natl. Acad. Sci. USA 77, 6851-6855).
- A similar method as above is described in the DE-patent publication No. 2,950,295 and in the Lancet Oct. 10, 1982, pp. 765-767. In the DE-patent publication No. 2,950,295 a method for the production of a radioactively labelled DNA probe by aid of recombinant DNA techniques is described.
- This DNA probe is made from the hepatitis B virus. In the Lancet the use of this probe in a method to detect hepatitis B virus is described. In this method the DNA from the sample is transferred to a nitrocellulose filter and the fixed hepatitis DNA from the sample is detected by using the probe as a reagent.

- Because two reagents are used, our method is much more specific than the method described above and makes it possible to construct different kits by aid of which different microbes or microbial groups can be detected from the same sample without disturbing the specificity of the test

3

for each microbe or microbial group in question. In the method according to the invention, two different nucleic acid fragment reagents - one attached to a solid carrier, the other labelled with a suitable label - are used and the sample DNA is in the liquid state.

The invention described herein is based on the sandwich hybridization technique (Dunn, A.R. and Hassell, J.A. (1977) Cell 12, 23-36), which simplifies the handling of the sample and the detection of the hybrid. For this reason the technique is particularly suitable for diagnostic use.

In the method described in this invention all the desired microbes or microbial groups can be identified from one and the same sample that contains denatured single-stranded nucleic acid strands of the microbes or microbial groups to be identified, without sample division. The method requires two nucleic acid reagents for each microbe or group of microbes to be identified. The reagents are two separate nucleic acid fragments derived from the genome of the microbe to be identified, which have no sequence in common, but preferably are situated close to each other in the genome. The reagents can be prepared directly from the microbial genomes or by using the established recombinant DNA techniques. Of the two nucleic acid fragments, one is fixed to a solid carrier, preferably a nitrocellulose filter, after being denatured, and the other also in single-stranded form is labelled with a suitable label. When these nucleic acid reagents, two different reagents for each microbe or microbial group to be identified, are placed in contact with the single-stranded nucleic acids to be identified in the sample, the nucleic acids anneal to the complementary nucleic acid fragments on the solid carrier. The hybrids thus formed on the carrier become labelled after annealing to the labelled complementary nucleic acid fragments. The labelled nucleic acid fragments do not alone

hybridize to the nucleic acid fragments attached to the carrier, but only to the corresponding single-stranded nucleic acids originating from the sample. Thus only those carriers to which the complementary nucleic acids from the sample have hybridized can become labelled. These carriers are easily washed and the label measured by established methods.

The method according to this invention can in principle be used for the identification of all either DNA- or RNA-containing organisms, such as viruses, bacteria, fungi and yeasts. The method has the specific advantage of permitting identification of all the bacteria and viruses possibly in question at the same time and from the same sample, regardless of whether the microbes contain DNA or RNA. By suitable combination of reagents it is possible to develop "kits" such as each microbe to be identified has its own solid carrier equipped with a tag and labelled nucleic acid reagent. All the filters included in the reagent combination can be added to the sample simultaneously, along with the labelled nucleic acid reagents. When hybridization has taken place, the solid carriers are washed and their labelling is measured. The only carrier to become labelled is the one containing sequences complementary to the microbial genome in the investigated sample.

The combination of method and reagents can be used eg. in medical microbiology, veterinary microbiology, food hygiene investigations and microbial diagnostics of plant diseases. Suitable sample materials are eg. all animal and plant tissue homogenates, patient secretions such as blood, faeces and nasal and urethral mucous. It can be estimated that the method is sufficiently sensitive to detect the microbe levels normally present in clinical samples. Preliminary enrichment of the microbe present in the sample by cultivation is of course possible before the identification

test and in some cases would be essential. The method is also suitable for the investigation of samples from which the microbe can no longer be cultivated, but which contain considerable amounts of microbial debris (eg. after the commencement of antibiotic treatment), or when cultivation of the microbe is particularly laborious and difficult (eg. anaerobic bacteria, which are present in large numbers in suppurative samples in the case of infections caused by anaerobes.

The method could be adapted in the form of the following reagent combinations, "kits", parts of which could of course be used separately:

15 Respiratory infections:

- a) Bacteria: β -haemolytic streptococci (A-group), Haemophilus influenzae, Pneumococci, Mycoplasma pneumoniae, mycobacteria
- b) Viruses: Influenza A, Influenza B, Parainfluenza 1-3
Respiratory syncytial virus, adenoviruses, corona viruses, rhinoviruses

25 Diarrhoeas:

- a) Bacteria: salmonellae, shigellae, Yersinia enterocolitica, enterotoxigenic E. coli, Clostridium difficile, campylobacteria
- b) Viruses: rotaviruses, parvoviruses, adenoviruses, enteroviruses

Veneral diseases:

- 5 a) Bacteria: Neisseria gonorrhoeae, Treponema pallidum,
Chlamydia trachomatis
- b) Viruses: Herpes simplex-virus
- c) Yeasts: Candida albicans
- 10 d) Protozoa: Trichomonas vaginalis

Sepsis:

- 15 a) Bacteria: β -haemolytic streptococci (A-group),
pneumococci, enterobacteria as a single group

Food hygiene:

- 20 a) Bacteria: Salmonella and Clostridium perfringens

Depending on the choice of reagents the specificity of the test can be limited to a defined microbial genus (eg. salmonellas) or to a whole microbial group eg. enterobacteriaceae by choosing identifying reagents from the area of a common gene.

25

The nucleic acid reagents required in the sandwich hybridization technique described in this invention are produced by recombinant DNA technology. In the following the reagent production and test procedure for example 1 are described.

30

Reagents

- 35 Adenovirus type 2 (strain deposited at KTL, i.e. the National Public Health Institute, Helsinki) was cultivated

and purified and DNA was isolated (Pettersen, U. and Sambrook, J. (1973) J. Mol. Biol. 73, 125-130) (referred to hereafter as Ad₂-DNA). The DNA was digested with BamHI-restriction enzyme (BRL, i.e. Bethesda Research Laboratories) that cuts the DNA into four reproducible fragments. Of these four fragments two were inserted to the BamHI-site of the vector plasmid pBR 322 (BRL) with the aid of T4-ligase (BRL). (The fragments were not separated before ligation, but the insert added to the plasmid was in each case identified only after cloning). Subsequently the bacterial host (E. coli HB101 (K12) gal⁻, pro⁻, leu⁻, hrs⁻, hrm⁻, recA, str^r, F⁻) (obtained from KTL) was transformed with the plasmid DNA composed of recombinant plasmids, i.e. molecules that had accepted fragments of the adenovirus-DNA (Cohen, S.N. et al. (1972) Proc. Natl. Acad. Sci. USA 69, 2110-2114). Among the transformed bacterial clones such as most probably contained the recombinant plasmid were chosen. Ampicillin and tetracycline resistance genes are transferred to the bacterium by the pBR322-plasmid (Bolivar F. et al. (1977) Gene 2, 95-113). Bacteria containing the recombinant plasmid are, however, sensitive to tetracycline, because the BamHI-restriction site is within the tetracycline gene and the foreign DNA inserting into this region destroys the gene. The insert of the plasmid was characterized after plasmid enrichment by determining the size of the restriction fragments after BamHI digestion using agarose gel electrophoresis. The adjacent BamHI D- and C-fragments of the Ad₂-DNA (cf. gene map) were chosen as reagents (Söderlund, H. et al. (1976) Cell 7, 585-593). The desired recombinant plasmids, Ad₂C-pBR322, KTL No. E231 and Ad₂D-pBR322, KTL No. EH230, were cultivated and purified as has been described in the literature (Clewett, D.B. and Helinski, D.R. (1969) Proc. Natl. Acad. Sci. USA 62, 1159-1166).



The recombinant plasmid Ad₂D-pBR322 was used as the filter reagent. It is not necessary for the present application to remove the plasmid sequences, because the sample does not contain pBR322-sequences. However, for radioactive labelling the nucleic acid was separated from pBR322-DNA after BamHI-digestion with the aid of agarose gel electrophoresis. The C-fragment was isolated from LGT-agarose (Marine Colloids, Inc.) by phenol extraction or electro-elution (Wieslander, L. (1979) Anal. Biochem. 98, 305-309) and concentrated by ethanol precipitation.

It is particularly expedient to subclone the nucleic acid fragment chosen for labelling in a separate vector, in order to avoid the hybridization background resulting from the direct hybridization with the filter of the residual plasmid sequences, contaminating the labelled nucleic acid reagent. The single-stranded DNA-phage M13 mp7 (BRL), to which DNA fragments obtained by BamHI-digestion can easily be transferred, could be used as an optimal vector (Messing, J. et al. (1981) Nucleic Acids Res. 9, 309-323).

Attachment of DNA to the filter

The recombinant plasmid Ad₂D-pBR322 was denatured to a single-stranded form and nicked randomly at several sites by treatment with 0.2 N NaOH (5 min. 100°C), whereafter the DNA was chilled and, immediately prior to transference to the filter, neutralized and pipetted to the transfer solution, 4 x SSC medium on ice (SSC = 0.15 M NaCl, 0.015 M Sodium citrate). The filters (Schleicher and Schüll BA85 nitro-cellulose) were thoroughly wetted in 4 x SSC solution (about 2 h) before the application of DNA. The DNA is attached to the filter in a dilute solution (0.5-1.0 µg/ml) by sucking the solution through the filter in a weak vacuum. The filter is capable of absorbing DNA up to about 180 µg/cm² (Kafatos, F.C. et al. (1979) Nucleic Acids Res. 7, 1541-1552). We have

used DNA-concentrations of between 0.5 µg DNA/2.5 cm diameter of filter and 1.0 µg DNA/0.7 cm diameter of filter. After DNA-filtration the filters are washed in 4 x SSC, dried at room temperature and finally baked in vacuum oven at 80°C for 2 h, after which the DNA on the filters remains stable and the filters can be stored for long periods at room temperature (Southern, E.M. (1975) J. Mol. Biol. 98, 503-517).

10 Labelling of the radioactive nucleic acid fragment

The radioactive label used was the ¹²⁵I-isotope. This isotope can be detected using γ-counters, which are available in most large laboratory units. The half-life of the isotope is 60 days, for which reason the utilization period of ¹²⁵I-labelled reagents is about 4 months.

"Nick-translation" labelling

20 The principle of this method is to displace one of the nucleotides in the nucleic acid with a radioactive one, upon which the whole DNA molecule becomes labelled. This is carried out according to the method published by Rigby, P.W.J. et al. (J. Mol. Biol. (1977) 113, 237-251). In the
25 reaction the DNA becomes radioactively labelled when the solution contains ¹²⁵I-labelled deoxynucleoside triphosphate as substrate, in this case ¹²⁵I-dCTP (Radiochemical Centre, Amersham: >1500 Ci/mmol). Under optimal conditions a specific activity of 10⁹ cpm/µg DNA can be obtained. The
30 labelled DNA is purified from nucleotides remaining in the reaction mixture by simple gel filtration, eg. using BioGel P30 (BioRad).

Other labelling methods

The single-stranded nucleic acid reagent produced in M13 mp7-phage is labelled by chemical iodination, in which the reactive ^{125}I is added covalently to the nucleic acid (Commenford, S.L. (1971) Biochemistry 10, 1993-2000, Grosz, J.M. and Wetmur, J.G. (1974) Biochemistry 13, 5467-5473). Alternatively, the nucleic acid can be made radioactive by endlabelling with radioactive nucleotides by the terminal transferase (Roychoudhury, R. and Wu, R. (1980) Meth. Enzymol. 65, 43-62).

The reagent preparation described above relates to microbes of which the genetic material is in the form of DNA. In the case of RNA viruses the cloning of genome fragments takes place in such a way that at first a DNA copy (cDNA) of the virus RNA has been made with the aid of reverse transcriptase, followed by DNA-polymerase to copy the second DNA strand, thereafter the DNA is cloned as described above (Salser, W. (1979) in Genetic Engineering, Ed. A.M. Chakrabarty, CRC Press, pp. 53-81).

The most suitable cloning method is chosen depending on the microbe used. The hosts as well as the vectors can vary. Possibilities include the λ -phage as vector, other plasmids, cosmids, cloning eg. in Bacillus subtilis bacteria, etc. (Recombinant DNA, Benchmarck Papers in Microbiology, Vol. 15, Eds. K.J. Denniston and L.W. Enqvist, Dowden, Hutchinson and Ross, Inc. (1981); Ish-Horowicz, D. and Burke, J.F. (1981) Nucleic Acids Res. 9, 2989-2998).

Performance of the test

Sample treatment

- 5 The microbial nucleic acid to be investigated must be released from the microbe itself and also from the infected cells, after which it must be denatured to the single-stranded form. Virus genomes can be liberated by treating the sample material with 1 % sodium dodecylsulphate (SDS)
- 10 and destroying the proteins protecting the genome by proteinase K-treatment (1 mg/ml, 37°C, 60 min). Bacterial samples must in addition be broken down using lysozyme- and EDTA-treatment.
- 15 If the sample contains large quantities of viscous high-molecular weight cellular-DNA, this must be sheared at a few sites in order to reduce its viscosity, eg. by sonication or by passing the sample a few times through a fine needle.

20 Hybridization

- Hybridization takes place eg. in 50 % formamide (deionized, stored at -20°C), in 4 x SSC Denhardt solution (Denhardt, D.T. (1966) Biochem. Biophys. Res. Commun. 23, 641-646)
- 25 containing 1 % SDS and 0.5 mg/ml DNA (salmon sperm or calf thymus) at 37°C and usually overnight for 16-20 hours. The filters chosen for the test are incubated in a suitable vessel, to which the hybridization mixture is added and the hybridization is started. The hybridization mixture contains
- 30 (a) the pretreated sample to which is added the radioactive nucleic acid reagent(s) that have been denatured together by boiling for 5 minutes followed by quick cooling at 0°C; (b) concentrated formamide-, SSC- and Denhardt-solutions, which are pipetted to the denatured and cooled nucleic acid
- 35 mixture (a). After mixing, the hybridization mixture is pipetted to the filters in the hybridization vessel. After

hybridization the filters are carefully washed and counted individually in the γ -counter.

5 The invention is clarified in the following with the aid of some practical examples.

Example 1

10 Detection of adenovirus by the sandwich hybridization method (Table 1)

The details of the test are clarified in the text to Table 1. The sandwich hybridization method can detect virus-DNA from a solution, but the viral genome can equally well be
15 detected from infected cells.

The hybridization background is measured in a tube containing only the filter and the labelled nucleic acid reagent, without the sample. The background is caused by the
20 pBR322 sequences occurring in the labelled nucleic acid reagent. These sequences hybridize directly with the filter without the sample mediating it. The filters containing calf thymus and no DNA are used in the test as controls, indicating on the one hand the specificity of hybridization
25 and on the other the level of the nonspecific background arising eg. from insufficient washing.

In the following tables the background due to the reagents has been subtracted from the cpm-values hybridized to the
30 filters.

Table 1Adenovirus test

Sample	Filters (cpm)		
	Adeno 1)	Calf thymus 2)	Blank 3)
Adenovirus type 2-DNA (BRL) (500 ng)	9000	49	-
HeLa-cells (6 x 10 ⁵) infected with adenovirus	8200	-	-

Filters:

- 15 1) Ad₂D-pBR322-plasmid, 2 µg
 2) Calf thymus DNA 1.µg (Boehringer Mannheim)
 3) Blank (no DNA)

Labelled nucleic acid reagent:

- 20 Ad₂-BamHI C-fragment, purified, specific activity
 90 x 10⁶ cpm/µg (200 000 cpm ¹²⁵I/reaction)

Hybridization:

- 50 % formamide, 4 x SSC
 25 Denhardt solution, containing 0.5 mg/ml salmon sperm DNA and
 1 % SDS, 37°C, 16 h

Washing:

- 0.1 % SSC, room temperature, 40 min

Samples:

Adenovirus type 2 DNA (BRL)

Infection with type 2 adenovirus took place in HeLa-cells.

The cells were then disrupted by treatment with 1 % SDS,

- 35 followed by digestion with 1 mg/ml proteinase-K-enzyme
 (Sigma) for 30 min 37°C. Before denaturation the sample was

passed through a fine needle. The values appearing in the table have been corrected by subtraction of the reagent background, obtained by carrying out a similar hybridization but without sample.

5

Example 2

Detection of an RNA-virus with the aid of sandwich hybridization (Table 2)

10

The model RNA-virus used was the Semliki Forest virus (prototype strain, obtained from the London School of Hygiene and Tropical Medicine), of which the genome is single-stranded RNA. Using the virus genome as a template cDNA was produced, which was cloned into the PstI site of pBR322 plasmid as described by Garoff et al. (Proc. Natl. Acad. Sci. (1980) USA 77, 6376-6380). The recombinant plasmid thus obtained is pKTH312 KTL No. EH 232. The insert of this plasmid originating from the virus genome is about 1400 nucleotides long and is from the structural protein area, approximately from nucleotide 200 to nucleotide 1600 when numbering is started from the beginning of the structural genes (Garoff, H. et al. 1980). For the production of reagent the whole recombinant plasmid pKTH312 was linearized with EcoRI restriction enzyme (BRL) (the sequence originating from the Semliki Forest virus does not contain recognition sites for the EcoRI-enzyme), and the linearized plasmid was cut into two fragments using XhoI-enzyme (BRL). The restriction site of the latter was located with the Semliki Forest virus sequence. The larger EcoRI-XhoI-fragment A (about 3900 base pairs) was attached to the filter and the smaller fragment B (about 1850 base pairs) was labelled with ^{125}I using the nick translation technique.

15

Both free Semliki Forest virus and virus-infected cells were used as samples in this test. In both cases the virus-specific nucleic acids of the sample were composed entirely of RNA.

5

Table 2

Detection of Semliki Forest virus with the aid of the sandwich hybridization method

10

Sample	Filters (cpm)		
	Semliki Forest virus 1)	Calf thymus 2)	Blank 3)
15 Semliki Forest virus 30 µg	3340	-	33
20 Cells infected with Semliki Forest virus (5 x 10 ⁵)	2698	8	10
Non-infected cells	10	5	8

Filters:

- 25 1) EcoRI-XhoI-fragment A (1.2 µg) of the pKTH312 plasmid
 2) Calf thymus DNA 1 µg
 3) Blank (no DNA)

Labelled nucleic acid reagents:

- 30 EcoRI-XhoI-fragment B of the plasmid pKTH312, specific activity 90 x 10⁶ cpm/µg DNA (200 000 cpm ¹²⁵I/reaction).

Hybridization:

As described in Table 1

35

Washing:

As described in Table 1

Samples:

- 5 Semliki Forest virus (30 µg) was disrupted with SDS before the test. The infected cells were handled as described in Table 1. The infection with Semliki Forest virus was carried out in BHK-21 cells.
- 10 The values given in the table have been corrected for reagent background, obtained from a similar hybridization without sample.

Example 3

15

A virus sample in which the viral messenger RNA is detected with the aid of the sandwich hybridization method (Table 3)

- The sandwich hybridization reagents were produced from SV40-virus DNA (BRL) by cutting the DNA into two parts using PstI-enzyme (Boehringer Mannheim) as described by Lebowitz and Weissman (Curr. Topics in Microbiol. Immunol. 87, 43-172) and the fragments were isolated and purified by agarose gel electrophoresis. Fragment A (4000 base pairs) was radioactively labelled with ¹²⁵I by nick translation and fragment B (1220 base pairs) was attached to the filter.
- 20
- 25

- The DNA fragments were chosen so that each contained areas coding for both early and late messengers. Thus fragment B contains about 700 bases from the structural protein gene VP1 and over 600 bases from the gene for early messengers. Because the DNA of SV40 virus is in itself a covalently closed ring, it cannot be detected by the test before linearization. Therefore, when infected cells are used as the sample it is possible to test how well the method is adaptable to the detection of RNA copies of the viral
- 30
- 35

genome. As can be seen from the results in Table 3, the test is excellently suited to the investigation of infected cells. The table also demonstrates that the same reagents can be used to investigate both the viral DNA and mRNA made from it.

Table 3

Detection of SV40-virus by the sandwich hybridization technique

Sample	Filters (cpm)		
	SV40 1)	Calf thymus 2)	Blank 3)
<u>Test 1</u>			
SV40 viral DNA (50 ng) (linearized)	20061	159	104
No sample	-	-	-
<u>Test 2</u>			
CV1-cells infected with SV40-virus 40 h after infection (10^6 cells)	30814	294	580
Non-infected cells	-	-	-

Filters:

- 1) The shorter fragment PstI B (0.2 μ g) of the circular SV40-virus DNA digested with PstI-restriction enzyme
- 2) Calf thymus DNA 1 μ g
- 3) Blank (no DNA)

Labelled nucleic acid reagent:

- The longer PstI A-fragment of the SV40-virus DNA, specific activity 28×10^6 cpm/ μ g DNA (200 000 cpm 125 I/reaction)

Hybridization:

As described in Table 1

The hybridization time is 40 h

5 Washing:

As described in Table 1

Samples:

10 SV40-virus DNA (BRL) was linearized with EcoRI restriction enzyme (BRL). CV1-cells (Biomedical Centre, Uppsala University) were infected with SV40-virus (obtained from Janice Y. Chou and Robert G. Martini, NIH, Bethesda) and the cells were harvested 40 h after infection. Treatment of the sample was as described in Table 1.

15

The values presented in the table have been corrected for reagent background, obtained from a similar hybridization carried out without sample.

20 Example 4Detection of Bacillus amyloliquefaciens by sandwich hybridization (Table 4)

25 The reagents were fragments of the α -amylase gene of B. amyloliquefaciens E 18 (Technical Research Centre of Finland, VTT), which were isolated for the purpose of this test from the recombinant plasmid pKTH10 (Palva, I. et al. (1981) Gene, 15, 43-51) by treatment with restriction enzyme
30 and subsequent agarose gel electrophoresis. The fragments used for this test were the ClaI-EcoRI fragment area of the α -amylase gene (460 base pairs) (ClaI Boehringer Mannheim) and the EcoRI-BamHI fragment (1500 base pairs). The EcoRI-BamHI fragment was attached to the filter and the
35 ClaI-EcoRI fragment was radioactively labelled with ^{125}I by nick translation.

As can be seen from Table 4, the B. amyloliquefaciens in a sample was identifiable by sandwich hybridization on the basis of the single α -amylase gene. E. coli gave a negative result in this test (indistinguishable from the background).

5

Table 4

Bacterial diagnostics by sandwich hybridization

Sample	Filters (cmp)		
	α -amylase 1)	Calf thymus 2)	Blank 3)
pKTH10-plasmid-DNA (linearized) 1 μ g	5773	47	-
No sample	-	-	-
<u>E. coli</u> HB101 (109)	-	-	-
<u>Bacillus amylolique-</u> <u>faciens</u> (3 x 10 ⁹)	3377	-	-
<u>Bacillus amylolique-</u> <u>faciens</u> (10 ⁹)	2871	-	-

Filters:

- 1) The EcoRI-BamHI fragment of the α -amylase gene from plasmid pKTH10, 0.35 μ g
- 30 2) Calf thymus DNA, 1 μ g
- 3) Blank (no DNA)

Labelled nucleic acid reagent:

- The ClaI-EcoRI fragment of the α -amylase gene from plasmid pKTH10, specific activity 35 x 10⁶ cpm/ μ g (200 000 cpm 125I/reaction)

Hybridization:

As described in Table 1

Washing:

5 As described in Table 1

Samples:

Bacterial samples were treated with lysozyme (67 µg/ml) for 30 min at 37°C; 5 mM EDTA was added to E. coli samples, too.

10 After the treatment SDS was added to all the samples (final concentration 2 %), which were then passed twice through a fine needle to reduce their viscosity before being denatured by boiling as described in the text relating to handling of samples.

15

The values appearing in the table have been corrected for reagent background, obtained from a similar hybridization without sample.

20 Example 5

An example of a reagent combination kit based on the sandwich hybridization method (Table 5)

25 The samples investigated in this test were cells infected by three viruses (adenovirus, SV40 virus and Herpes simplex virus) and a sample containing Bacillus amyloliquefaciens bacteria. The following reagents were all simultaneously added to each sample, 5 filters, each containing one type of

30 DNA from SV40 virus, adenovirus, Bacillus amyloliquefaciens α-amylase gene and calf thymus, as well as a filter containing no DNA at all; in addition 200 000 cpm of each of the following labelled nucleic acid reagents: SV40 virus-, adenovirus- and α-amylase gene DNA-reagent.

35

Our example shows that it is possible, without division or dilution of the sample, to investigate simultaneously a suitable series of microbes by adding the reagent combination to the sample. The sample may contain both viral and bacterial nucleic acid. The filters can be recognized by a sign (= mark, tags), which identifies the sequence it contains and tags, which microbe was attached/hybridized to it. The signs can be numbers or letters, eg. 1 or SV40 2 or Ad etc. or other signs as * for SV40 or Δ for AD or O for Bacillus.

Table 5A kit based on the filter hybridization technique

Sample	Filters (cpm)				
	SV40 1)	Adeno 2)	α-amylase 3)	Calf thymus 4)	Blank 5)
Cells infected with SV40 virus (10 ⁶)	<u>18390</u>	2	13	22	31
Cells infected with adenovirus type 2 (6x10 ⁵)	-	<u>8750</u>	5	13	-
Cells infected with Herpes simplex virus (10 ⁶)	-	-	-	5	13
<u>Bacillus amylo-</u> <u>liquefaciens</u> (10 ⁹)	15	8	6500	16	5
Non-infected cells	-	-	-	-	-

Filters:

- 1) As in Table 3
- 2) As in Table 1
- 3) As in Table 4
- 5 4) Calf thymus DNA, 1 μ g
- 5) Blank (no DNA)

Labelled nucleic acid reagents:

- SV40 virus as in Table 3
- 10 Adenovirus as in Table 1
- α -amylase gene as in Table 4

Hybridization:

As in Table 1

15

Washing:

As in Table 1

Samples:

- 20 Cell samples infected with SV40 virus and adenovirus have been described in Tables 3 and 1, respectively.

- 10⁶ Vero cells were infected with Herpes simplex virus type 1. The cells were harvested 20 h post infection as
- 25 cytopathic effect could be observed. The sample was treated as described for adenovirus infected cells (Table 1).

Bacillus amyloliquefaciens sample:

As in Table 4

30

The values in the table are corrected for reagent background, obtained by carrying out a similar hybridization without sample.

Example 6Detection of Escherichia coli by sandwich hybridization
(Table 6)

5

The reagents were prepared from the ompA-gene (outer membrane protein A -gene) of Escherichia coli.

10 The hybrid plasmids pKTH40 and pKTH45, used as starting material, were prepared from the pTU100 plasmid described by Henning et al. (1979) Proc. Natl. Acad. Sci. USA 76, 4360-4364.

15 The plasmid pKTH45 (deposited at KTL, i.e. National Public Health Institute, Helsinki No. ...), used as a filter reagent, was composed of 740 base pairs from 5' -terminal end of the ompA-gene inserted into the pBR322-plasmid.

20 The plasmid pKTH40 contains 300 base pairs from the 3' -terminal end of the ompA-gene and the immediately following 1700 base pairs from the genome of E. coli. The pKTH40 plasmid was cleaved with the BamHI restriction enzyme to receive the DNA fragment of E. coli, which contains the 1700 base pairs mentioned above. This fragment was transferred to
25 the single-stranded bacteriophage M13mp7 according to the methods described by Messing et al. (1981), Nucl. Acids Res. 9, 309-321, Heidecker et al. (1980), Gene 10, 69-73, Gardner et al. (1981), Nucl. Acids Res. 9, 2871-2888. The recombination-phage mKTH1207 (deposited at KTL No. ...) was
30 labelled with ¹²⁵I-isotope as described on page 10 under the heading "Other labelling methods" and was used as a probe in the sandwich hybridization method.

35 DNA from disrupted E. coli cells, as well as isolated, purified DNA from E. coli, can be detected by sandwich hybridization as shown in Table 6.

Table 6Detection of Escherichia coli by sandwich hybridization

5	Sample	Filters (cpm)		
		ompA 1)	Calf thymus 2)	Blank 3)
10	<u>E. coli</u> K12 HB101 DNA a) 2×10^7	282	-	-
	<u>E. coli</u> K12 HB101 DNA a) 2×10^8	2206	-	-
15	<u>E. coli</u> K12 HB101 Cells b) 2×10^7	1113	-	-
	<u>E. coli</u> K12 HB101 Cells b) 2×10^8	2327	12	5
20	a) number of DNA-molecules b) number of cells			

Filters:

- 1) pKTH45 plasmid 1.088 μg (2×10^{11} molecules)
 25 2) Calf thymus DNA 1.088 μg
 3) Blank (no DNA)

Labelled nucleic acid reagents:

- mKTH1207, specific activity 8×10^7 cpm/ μg DNA
 30 (200.000 cpm/reaction)

Hybridization:

- 4 x SCC, 1 x Denhardt solution without BSA (bovine serum albumin), 0.25 % SDS, 200 $\mu\text{g/ml}$ Herring sperm DNA, 17.5 h,
 35 +65°C

Washing:

As described in Table 1

Samples:

- 5 E. coli K12 HB101 -DNA was isolated according to the Marmur-method described by Marmur (1961) J. Mol. Biol. 3, 208-218. DNA was denatured at 7mM NaOH, +100°C, 5 min.

- 10 The cells were treated with lysozyme (500 µg/ml), EDTA (70 mM +37°C, 30 min), SDS (0.25 %, +65°C) and the free DNA was denatured by boiling at 14 mM NaOH, +100°C, 5 min).

- 15 The values presented in the table have been corrected for reagent background obtained from a similar hybridization without sample.

PATENT CLAIMS

1. A microbial diagnostic method, based on sandwich hybridization of nucleic acids on a solid carrier, to
5 identify microbes or microbial groups, in which method two different nucleic acid reagents with no sequences in common, but preferably situated close to each other in the genome of the given microbe or microbial group, are used for the identification of that microbe or microbial group in a given
10 sample, one of the nucleic acid reagents being a single-stranded nucleic acid fragment attached to a solid carrier and the other being a single-stranded nucleic acid fragment labelled with a suitable marker, the method being
c h a r a c t e r i z e d in that the nucleic acid
15 reagents are brought into contact with the nucleic acids of all the microbes or microbial groups in the undivided sample, where the sample nucleic acids are first, by known methods, rendered single-stranded, upon which the sample nucleic acids hybridize to the complementary nucleic acid
20 fragments, themselves attached to the solid carrier and the carrier-attached hybrids become labelled due to the subsequent annealing of labelled nucleic acid fragments to the sample nucleic acid fragments, after which the carrier-attached label is measured by established methods.
- 25
2. A reagent combination to be used in the method according to patent claim 1, c h a r a c t e r i z e d in that it includes two nucleic acid reagents for each microbe or microbial group to be identified, and the necessary two
30 different nucleic acid fragments, which have been made from different parts being preferably near each other of the microbe or microbial group for the reagents, have been produced either directly from the microbial genome or using established recombinant DNA techniques, and these group- or
35 species-specific nucleic acid fragments have been rendered

single-stranded, and one has been attached to a solid carrier and the other labelled with a suitable marker.

3. A reagent combination according to claim 2,
5 characterized in that for the identification of adenovirus it includes the adenovirus recombinant plasmid Ad₂DpBR322 as a solid carrier attached nucleic acid reagent and the adenovirus Ad₂-BamHI C-fragment as a labelled nucleic acid reagent.
- 10 4. A reagent combination according to claim 2, characterized in that for the identification of Semlike Forest virus it includes the EcoRI-XhoI fragment A of the pKTH312 plasmid as a solid carrier attached nucleic
15 acid reagent and the EcoRI-XhoI fragment B of the pKTH312 plasmid as a labelled nucleic acid reagent.
- 20 5. A reagent combination according to claim 2, characterized in that for the identification of SV40 virus it includes the PstI B fragment of SV40 virus as a solid carrier attached nucleic acid reagent and the PstI A fragment of SV40 virus as a labelled nucleic acid reagent.
- 25 6. A reagent combination according to claim 2, characterized in that for the identification of Bacillus amyloliquefaciens it includes the EcoRI-BamHI fragment of the α -amylase gene of the Bacillus amyloliquefaciens plasmid pKTH10 as a solid carrier attached
30 nucleic acid reagent and the ClaI-EcoRI fragment of the α -amylase gene of the Bacillus amyloliquefaciens plasmid pKTH10 as a labelled nucleic acid reagent.
- 35 7. A reagent combination according to claim 2, characterized in that E. coli is identified using the plasmid pKTH45 as a solid carrier attached nucleic

acid reagent and the recombination-phage mKTH1207 as a labelled nucleic acid reagent.

8. A reagent combination according to claims 2, 3, 5 and 6,
5 c h a r a c t e r i z e d in that for the identification
and distinction of different microbes, viruses as well as
bacteria, such as adenovirus, SV40 virus and Bacillus
anyloliquefaciens, from the same sample containing nucleic
acids originating from different microbes, the combination
10 includes the Ad₂DpBR322 recombinant plasmid of adenovirus,
the PstI B fragment of SV40 virus and the EcoRI-BamHI
fragment of the α -amylase gene of the Bacillus
anyloliquefaciens plasmid pKTH10 as solid carrier-attached
nucleic acid reagents, and the Ad₂-BamHI C fragment of
15 adenovirus, the PstI A fragment of SV40 virus and the
ClaI-EcoRI fragment of the α -amylase gene of the Bacillus
anyloliquefaciens plasmid pKTH10 as labelled nucleic acid
reagents.

AMENDED CLAIMS

(received by the International Bureau on 07 March 1983 (07.03.83)).

(amended) 1. A microbial diagnostic method, based on sandwich hybridization of nucleic acids on a solid carrier, to identify microbes or microbial groups, in which method two different nucleic acid reagents with no sequences in common, but preferably situated close to each other in the genome of the given microbe or microbial group, are used for the identification of that microbe or microbial group in a given sample, one of the nucleic acid reagents being a single-stranded nucleic acid fragment attached to a solid carrier and the other being a single-stranded nucleic acid fragment labelled with a suitable marker, the method being characterized in that for the simultaneous identification of different microbes or microbial groups a plurality of nucleic acid reagents are brought into contact with the nucleic acids of all the microbes or microbial groups in the undivided sample, where the sample nucleic acids are first, by known methods, rendered single-stranded, upon which the sample nucleic acids hybridize to the complementary nucleic acid fragments, themselves attached to the solid carrier and the carrier-attached hybrids become labelled due to the subsequent annealing of labelled nucleic acid fragments to the sample nucleic acid fragments, after which the carrier-attached label is measured by established methods.

(amended) 2. A plurality of reagent combinations to be used in the method according to patent claim 1, characterized in that it includes two nucleic acid reagents for each microbe or microbial group to be identified, and the necessary two different nucleic acid fragments, which have been made from different parts being preferably near each other of the microbe or microbial group for the reagents, have been produced either directly from the microbial genome or using established recombinant DNA techniques, and these

group- or species-specific nucleic acid fragments have been rendered single-stranded, and one has been attached to a solid carrier and the other labelled with a suitable marker.

(amended) 3. A plurality of reagent combinations according to claim 2, characterized in that for the identification of adenovirus it includes the adenovirus recombinant plasmid Ad₂DpBR322 as a solid carrier attached nucleic acid reagent and the adenovirus Ad₂-BamHI C-fragment as a labelled nucleic acid reagent.

(amended) 4. A plurality of reagent combinations according to claim 2, characterized in that for the identification of Semlike Forest virus it includes the EcoRI-XhoI fragment A of the pKTH312 plasmid as a solid carrier attached nucleic acid reagent and the EcoRI-XhoI fragment B of the pKTH312 plasmid as a labelled nucleic acid reagent.

(amended) 5. A plurality of reagent combinations according to claim 2, characterized in that for the identification of SV40 virus it includes the PstI B fragment of SV40 virus as a solid carrier attached nucleic acid reagent and the PstI A fragment of SV40 virus as a labelled nucleic acid reagent.

(amended) 6. A plurality of reagent combinations according to claim 2, characterized in that for the identification of Bacillus amyloliquefaciens it includes the EcoRI-BamHI fragment of the α -amylase gene of the Bacillus amyloliquefaciens plasmid pKTH10 as a solid carrier attached nucleic acid reagent and the ClaI-EcoRI fragment of the α -amylase gene of the Bacillus amyloliquefaciens plasmid pKTH10 as a labelled nucleic acid reagent.

(amended) 7. A plurality of reagent combinations according to claim 2,

characterized in that E. coli is identified using the plasmid pKTH45 as a solid carrier attached nucleic acid reagent and the recombination-phage mKTH1207 as a labelled nucleic acid reagent.

(amended) 8. A plurality of reagent combinations according to claims 2, 3, 5 and 6, characterized in that for the identification and distinction of different microbes, viruses as well as bacteria, such as adenovirus, SV40 virus and Bacillus amyloliquefaciens, from the same sample containing nucleic acids originating from different microbes, the combination includes the Ad₂DpBR322 recombinant plasmid of adenovirus, the PstI B fragment of SV40 virus and the EcoRI-BamHI fragment of the α -amylase gene of the Bacillus amyloliquefaciens plasmid pKTH10 as solid carrier-attached nucleic acid reagents, and the Ad₂-BamHI C fragment of adenovirus, the PstI A fragment of SV40 virus and the ClaI-EcoRI fragment of the α -amylase gene of the Bacillus amyloliquefaciens plasmid pKTH10 as labelled nucleic acid reagents.

INTERNATIONAL SEARCH REPORT

International Application No PCT/FI82/00038

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC 3

C 12 Q 1/68, G 01 N 33/58

II. FIELDS SEARCHED

Minimum Documentation Searched *

Classification System

Classification Symbols

IPC 3

C 12 Q 1/68, 00, 16, 70, G 01 N 33/58, 60, 48, 50, 52

US C1

435:6, 5, 172

Documentation Searched other than Minimum Documentation
to the extent that such Documents are included in the Fields Searched *

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category ¹⁵	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	Chemical Abstracts Vol 93 (1981), abstract No 65229w, Methods Enzymol. 1980, 65 (Nucleic Acids Pt I) 468-78.	1
A	Chemical Abstracts Vol 79 (1973), abstract No 50606d, Proc. Nat. Acad. Sci. USA 1973, 70(6), 1697-1700.	1
A	GB, A, 2 019 408 (INSTITUT PASTEUR) 31 October 1979	1
A	GB, A, 2 034 323 (INSTITUT PASTEUR) 4 June 1980	1
P	US, A, 4 302 204 (WAHL G M and STARK G R) 24 November 1981	1
E	DE, A1, 3 211 311 (BETHESDA RESEARCH LABORATORIES INC) 7 October 1982	1
A	Derwent's Abstract No 85535B/47, SU 649 751	1, 7

* Special categories of cited documents: ¹⁴

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or priority date and not in conflict with the application but
cited to understand the principle or theory underlying the
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cannot be considered novel or cannot be considered to
involve an inventive step

"Y" document of particular relevance; the claimed invention
cannot be considered to involve an inventive step when the
document is combined with one or more other such docu-
ments, such combination being obvious to a person skilled
in the art.

"Z" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search *

1982-12-29

Date of Mailing of this International Search Report *

1983-01-13

International Searching Authority *

Swedish Patent Office

Signature of Authorized Officer ²⁰

C-O Gustafsson

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. 18
E	EP, A1, 0 062 286 (ALBERT EINSTEIN COLLEGE OF MEDICINE OF YESHIVA UNIVERSITY) 13 October 1982	1
E	EP, A2, 0 063 879 (YALE UNIVERSITY) 3 November 1982	1
A	J. Inf. Dis. Vol 142, No 6, pp 892-98, published December 1980 (MOSELEY S L et al) "Detection of Enterotoxigenic Escherichia coli by DNA Colony Hybridization".	1, 7
A	J. Clin. Microbiol. Vol 12, No 2, published August 1980 (BRAUTIGAM A R et al), "Rapid Typing of Herpes Simplex Virus Isolates by Deoxyribonucleic Acid: Deoxyribonucleic Acid Hybridization".	1